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(54) Title: METHOD AND DEVICE FOR RAPID DNA OR RNA SEQUENCING DETERMINATION BY A BASE ADDITION SEQUENCING SCHEME

(57) Abstract

A multiple base addition sequencing scheme (BASS) for the rapid sequencing of oligonucleotides (DNA and RNA) involving the steps of attaching a plurality of DNA or RNA strands to be sequenced to a coated support, adding a modified nucleotide, detecting the modified nucleotide, removing the blocking group and repeating the steps until the RNA or DNA is sequenced. Methods for improving and monitoring coupling efficiency are included. Two separate methods of automation using BASS are described. The first includes buffer reservoirs, two controllable valves, a detector and a plurality of reaction columns. The second includes a solid support, a plurality of buffer reservoirs, a detector means, a computer means and a movement means. Also described is a device for simultaneously sequencing a plurality of RNA or DNA sequences.

METHOD AND DEVICE FOR RAPID DNA OR RNA SEQUENCING DETERMINATION BY A BASE ADDITION SEQUENCING SCHEME

Field of the Invention

This invention relates to the field of rapid sequencing of oligonucleotides by the base addition method. More particularly, this invention relates to a method for determining DNA and RNA sequences without gel electrophoresis, utilizing different leaving and reporting groups attached to the 3'-hydroxyl position of the nucleotides.

Background

The analysis of primary DNA sequence is an important process for all kinds of biological studies. Two general approaches have been favored for DNA sequencing in the last ten years. These are the Maxam-Gilbert method of modification of specific DNA bases followed by chemical degradation at the modified sites, and the Sanger dideoxy termination method. These approaches have the common feature that a population of DNA molecules of different sizes is generated by the sequencing procedure, and therefore gel electrophoresis is required in order to resolve the sequencing products and to complete the analysis.

There have been several attempts to speed up and simplify the DNA sequencing procedures. Improvements include: (1) using fluorescent labels in place of radioisotopes, (2) "multiplex" mixing of different molecules prior to sequencing reactions followed by transfer of the sequencing gels to a membrane and hybridization with a specific probe to identify each element, (3) using mass spectrometry to detect the different bases, (4) using solid supports to entrap the DNA or RNA template, or (5) using different stable isotopes to detect bases. All of these modified procedures rely on the previously described Sanger or Maxam-Gilbert approaches, and therefore, have the component of gel electrophoresis.

Another approach which is different includes DNA sequencing by the hybridization approach. This approach relies on specific hybridization of short oligonucleotides to sequences bound to a solid support. In this method either the DNA to be sequenced or the numerous necessary oligonucleotide probes must be fixed to a solid support and repeated hybridizations carried out. This method is unproven and differs both in concept and design to the Base Addition Sequencing Scheme of the present invention.

An additional method which has been described measures the release of a group from each base as it is joined to a growing oligonucleotide. For example, inorganic phosphate is released from the 5'-terminus of a specific deoxyribonucleotide triphosphate and is detected as a substrate for the enzyme luciferase.

A further procedure that aims to speed up sequence analysis is a method that uses enzymatic degradation of single stranded DNA molecules in an apparatus that has a rapid flow of reagents past a template molecule fixed on a solid support.

The present invention provides a novel approach for DNA sequencing comprising repetitive cycles of enzyme catalyzed incorporation of nucleotides into a DNA template primer complex. The nucleotides are added at the first available position and are blocked from further extension at the 3'-hydroxyl terminus by a chemical group. Each cycle of addition is followed by chemical release of a blocking group and addition of the next base. The detection of the blocking group either in situ or after leaving reveals the DNA sequence.

Summary of the Invention

An object of the present invention is a rapid assay for sequencing oligonucleotides of DNA and RNA, an aspect of which is to accomplish oligonucleotide sequencing without gel electrophoresis.

An additional object of the present invention is a multiplex DNA and RNA sequencing method.

A further object of the present invention is methods of automation for rapid sequencing of a plurality of DNA or RNA molecules by a base addition sequencing scheme.

A further object of the present invention is a device for rapid sequencing of a plurality of DNA or RNA molecules by a base addition sequencing scheme.

Thus, in accomplishing the foregoing objects, there is provide in accordance with one aspect of the present invention a multiplex assay for determining the sequence of a strand of DNA or RNA, comprising the steps of attaching a plurality of DNA or RNA templates with annealed primers to a solid support; contacting the attached DNA or RNA templates with a reaction buffer, said buffer containing modified nucleotides and an enzyme to attach the modified nucleotides to the 3'-hydroxyl terminus of the primer attached to each template, wherein the modified nucleotides include a blocking group and a reporter, wherein the blocking group blocks the addition of further nucleotides to the primer and the reporter identifies which nucleotide has attached to the primer; removing the unreacted modified nucleotides by washing the reacted DNA or RNA with a first wash buffer; detecting the attached modified nucleotide added to each primer; removing the blocking group and reporter by contacting the DNA or RNA with a detaching buffer; washing the attached DNA or RNA with a second wash buffer to remove traces of the detaching buffer and repeating the above steps until the DNA or RNA has been sequenced.

In specific embodiments of the present invention, the solid support is selected from the group consisting of a Strepavidin/biotin coated surface, a derivatized photoactivatable glass, and a NH_2 binding group attached to glass.

Another aspect of the present invention is devices for simultaneously sequencing a plurality of DNA or RNA sequences. A first device comprises: a first reservoir for a detaching buffer; a second reservoir for a reaction buffer; a third reservoir for a washing buffer; a plurality of columns, each column including a solid support for attaching a DNA or RNA template with attached primers; a detector means; a fourth reservoir for a capping buffer; and a fifth reservoir for waste; said first, through fourth reservoirs being connected to the entrance ends of said columns through a first controllable valve to supply the appropriate buffer to said columns; said detector means, fifth reservoir and second reservoir being connected to the exit end of said columns through a second controllable valve to direct the outflow of the columns flow through the detector and into the waste reservoir and to recycle the reaction buffer.

A second device comprises: a solid support for attaching the plurality of DNA or RNA templates with annealed primers at discrete locations; a plurality of buffer reservoirs for holding reaction buffer, wash buffers, capping buffer and detaching buffer; and a detector means for identifying the addition of a nucleotide to the annealed primers; wherein said solid support, buffer reservoirs and detector means are relationally positioned on a movement means such that there are repeated cycles of the solid support with attached DNA or RNA sequentially contacting the reaction buffer to add a modified nucleotide to the primers, the first wash buffer to remove unreacted nucleotides, a capping buffer to prevent further elongation of unreacted template primers, the detector means to identify which nucleotide was added to each primer, the detaching buffer for removing a blocking group and reporter from the attached modified nucleotide, and the second wash buffer to remove traces of the detaching buffer.

Both devices can be automated by attaching them to a computer means for controlling the sequencing steps and recording the image from the detector means.

Other and further objects, features and advantages will be apparent and eventually more readily understood from a reading of the following specification and by reference to the accompanying drawings forming a part thereof where examples of the presently preferred embodiments of the invention are given for the purpose of disclosure.

Description of the Drawings

Figure 1 is a schematic representation showing four modified nucleotides which can be used in the present invention. Figure 1A shows adenosine 5' triphosphate with Leaving Group "A"; 1B shows guanosine 5' triphosphate with Leaving Group "G"; 1C shows cytosine 5' triphosphate with Leaving Group "C"; and 1D shows thymine 5' triphosphate with Leaving Group "T".

Figures 2A, 2B and 2C are a serial schematic representation showing a base addition sequence scheme for a single DNA template attached to a solid support with a flow through detector.

Figure 3 is a schematic diagram for the base addition sequence scheme of multiple templates bound to solid supports with flow through detectors.

Figures 4A, 4B and 4C are a serial schematic representation of a base addition sequence scheme analysis of a single DNA template attached to a solid support with in situ detection.

Figure 5 is a schematic of a device for base addition sequence scheme analysis.

Figure 6 is a diagram of a multiplex template support for the base addition sequence scheme analysis of multiple DNA templates with in situ detection.

Figure 7 shows a purine base (adenosine) with a photolabile blocking group at the 3'-terminus.

The drawings and figures are not necessarily the scale and certain features may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

Detailed description of the invention

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the inventions disclosed herein without departing from the scope and the spirit of the invention.

The term "modified nucleotide" as used herein is defined as a nucleotide which has a chemical group attached to the nucleotide. This chemical group blocks the addition of further nucleotides through the 3'-hydroxyl group of the modified nucleotide. Thus a modified nucleotide, once attached to a primer, prevents further elongation through the 3'-hydroxyl group of the sequence unless the blocking group is removed.

The term "blocking group" and "blocker" as used herein is defined as any chemical group which can be attached to a DNA or RNA nucleotide and which will allow the 5' end of the modified nucleotide to attach to a 3' end of another nucleotide but will not allow attachment of a nucleotide to the 3'-hydroxyl group of the modified nucleotide. The blocking group can attach directly to the 3'-hydroxyl group (protection group) or attach to another part of the nucleotide and prevent reaction with the 3'-hydroxyl group for example by steric hinderance. The blocking group's chemical structure can also include a reporter or marker moiety.

The terms "reporter" or "marker" as used herein are defined as any element or molecule moiety which can be attached to the nucleotide and which can be used to detect and identify specific nucleotides. Some specific examples of reporters or markers include metal ions, fluorescent dyes, radioisotopes, antibodies and chemiluminescence compounds.

Normally when sequencing DNA or RNA the four different nucleotides, 2'—deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine and 2'-deoxythymidine, are used. Each nucleotide can be identified because a different reporter will be attached to each nucleotide. The reporter can be any combination of the above molecular moieties. For example the reporter could be different fluorescent compounds or different radioisotopes or different metal ions or a mixture of any combination of fluorescent compounds, radioactive compounds, metal ions or antibodies. One skilled in the art will readily recognize that the selection of the combination of reporters depends on the detection means being utilized. Examples of molecules used in the present invention are shown in Figure 1 and Figure 7.

Certain characteristics are important in identifying those groups which are useful for the blocking of the 3'-hydroxyl group. The functional definition of a hydroxyl blocking group includes compounds (i) which react with the 3'-hydroxyl group and prevent further unwanted reactions; (ii) are easily removed; and (iii) upon removal leave the hydroxyl group intact and able to react with the 5' end of another nucleotide. In the present invention various hydroxyl blocking groups are available, including: (i) ethers which are generally removed by acid treatment; (ii) esters which are generally removed by alkali/base treatment; (iii) carbonates; and (iv) sulphonates. Some specific examples of blocking groups include Tetrahydropyranyl ether, 1-ethoxyethyl ether, trimethylsilyl ether, Bis (2-chlorethoxy) methyl ether. Further examples are known in the art and some are shown in "Protective Groups in Organic Synthesis" by Theodora W. Greene, John Wiley & Sons, New York, 1981. An example of photolabile group can be seen in Ohtsuha, et al., J. Am. Chem. Soc. 100:4210-8213 (1978).

An additional alternative blocking group and means for its detachment is a photolabile blocking group as shown in figure 7. This

modified nucleotide is accepted by the BST polymerase, and terminates further primer elongation after its addition due to the bonding of the blocking of the 3'-hydroxyl terminus. This blocking group can be entirely removed by a 120 second exposure to short wavelength UV light. Since the UV wavelength required for removal of the labile blocking group is different from the UV wavelength that can damage DNA, narrow bandwidth UV emitters or UV filters can be used to protect the DNA from UV damage.

The blocking groups which can be used are well known by those skilled in the art. The blocking groups generally are those which can be removed either by base, acid, salt, ions, a combination of salt and ions, or by the bombardment of light. The blocking group and reporter can be combined.

One embodiment of the present invention is the assay for determining the sequence of a strand of DNA or RNA. This assay is shown schematically in Figures 2 and 4. The assay includes attaching a plurality of DNA or RNA templates 13 with annealed primers 16 to a solid support 10; contacting the attached DNA or RNA templates with a reaction buffer, said buffer containing modified nucleotides and an enzyme to attach the modified nucleotides to the 3'-hydroxyl terminus of the primer 16 attached to each DNA or RNA, wherein the modified nucleotides include a blocking group and a reporter, wherein the blocking group blocks the addition of further nucleotides to the primer and the reporter identifies which nucleotide has attached to the primer; removing the unreacted modified nucleotides by contacting the reacted DNA or RNA template with a first wash buffer; detecting the attached modified nucleotide added to each primer; capping any unreacted template; removing the blocking group and reporter by contacting the DNA or RNA with a detaching buffer; contacting the attached DNA or RNA with a

second wash buffer to remove traces of the detaching buffer; and repeating the above steps until the DNA or RNA has been sequenced.

In this method the solid support can be selected from any group of solid supports known to the art to bind DNA or RNA. Some solid supports which are useful in the procedure include strepavidin/biotin coated surfaces, derivatized photoactivatable surfaces and NH_2 binding groups attached to glass. In the preferred embodiment biotinylated DNA or RNA templates are attached to strepavidin coated supports.

In the present assay each different type of nucleotide which is being added to the RNA or DNA primer has a specific reporter attached. This reporter can be measured while attached to DNA (in situ) or can be cleaved with the blocking group and then measured. In a preferred embodiment of the present invention the reporters are a fluorescent compounds which are measured in situ by activation by laser excitation 25 and recording of the emissions spectra detector 28.

The reaction buffer is designed to add a modified nucleotide to the primer which has been attached to the DNA or RNA template strand to be sequenced. The reaction buffer will contain the modified nucleotides and an enzyme to attach the modified nucleotides to the 3' hydroxyl terminus of the primer attached to each DNA or RNA template. One skilled in the art will recognize that a variety of buffers can be used. In the preferred embodiment the buffer is 25mM Tris pH 7.8, 50mM NaCl, 10mM $MgCl_2$. The enzymes used to attach the modified nucleotide to the primer strand for the sequence analysis can include any of the following T7, Taq, Klenow, T_4, Pol 1, DNA polymerase and reverse transcriptase.

A further aspect of this embodiment is a method to remove unblocked (unmodified) nucleotides which may contaminate the reaction buffer. A source of this contamination comes from a very small percentage of the modified nucleotides having the labile modifying (blocking) group spontaneously detach under normal condition, leaving an

unblocked nucleotide. To remove any contaminating unblocked nucleotides, a scavenger reaction may be included in the reaction buffer.

In one embodiment, decontamination may be accomplished by including in the reaction buffer, nucleotide specific scavenger primer-template complexes and any additional necessary enzyme to incorporate unblocked nucleotides present into the scavenger complexes. The scavenger complexes bond any unblocked nucleotides, and because the complexes are not attached to the solid support, the contaminating nucleotides are removed along with the complexes in the following washing step. Pretreatment of the reaction buffer can be performed either with the same enzyme(s) being used to add the modified nucleotides, or by other additional enzyme(s). For example, terminal transferase may be used as an additional enzyme.

The wash buffers used in the present invention can be of a variety of different types. When removing unreacted modified nucleotides the wash buffer is usually 25mM Tris pH 7.8; 20mM NaCl. The same wash buffer can be used to remove traces of the detaching buffer.

An additional aspect of this embodiment is a method for "capping" unreacted 3'-hydroxyl termini of the primed templates on the solid support. Failure to couple a modified nucleotide to a primed template during contact with the reaction buffer results in that template's primer elongating out of sync with the rest of the template primers for the remainder of the sequencing procedure. This out of sync elongation causes a loss of fidelity of the signal from that particular template position and an overall loss of sensitivity.

To prevent unreacted template primers on the solid support from further elongation, a capping step may be incorporated into the assay cycle. Such a step may be inserted in the cycle after the addition of the modified nucleotides, but before the removal of blocking groups. The capping step comprises contacting the solid support with a capping buffer.

A capping buffer comprises an appropriate buffer containing a free molecular blocker (capping agent) and the necessary reagents under conditions to stably bond the molecular blocker (capping agent) to the template primer, and prevent further primer elongation. A capping agent may consist of stably blocked nucleotides or dideoxynucleotides.

An advantage of the capping step is its utility in monitoring the overall coupling efficiency of the BASS process. To monitor overall coupling efficiency of the sequencing process, the capping reagent can also contain a non-labile marker moiety. As an element of the capping reagent, this marker remains on the on the template primer throughout subsequent sequencing cycles. By detecting and monitoring the presence and accumulation of capped template primers, the overall efficiency of the sequencing procedure can be monitored. Such information is useful for overall reagent economy, for adjusting reagent and reaction parameters (e.g., concentrations, time, temperatures) for optimizing the sequencing procedure at every cycle, for determining the maximum number of repeat cycles a for a given sequencing procedure. A further particular advantage is that a capping reagent marker is an aspect of the present invention that embodies great utility in the automation of the BASS method. The presence and accumulation of the capping agent marker may be monitored in situ by a detector connected to automated control means, such as a computer. The signal from the capping agent marker via the detector can be used to determine the overall coupling efficiency, and provide the signal to a computer, for example, which can adjust the metering of reagents, reaction times and temperature to optimize overall BASS performance, as well as to terminate drastically failed sequencing procedure.

The detaching buffer is used to remove the blocking group and reporter from the modified nucleotide so that the next nucleotide can be added. Detaching buffers are well known in the art and depend on which

blocking group is being used. Examples of detaching buffers are given in "Protective Groups in Organic Synthesis" by Theodora W. Greene, John Wiley & Sons, New York, 1981. In using the detaching buffer one skilled in the art will readily recognize that, under the optimum conditions, the buffer must not be too strong of an acid or base so that the enzymes necessary to extend the chain are not destroyed or that the nucleotides are not hydrolyzed or altered. Further, too much base or heat will cause the strands to melt apart. Additionally, the enzyme is greatly affected by both salt and pH. On the other hand, if the conditions affect the enzyme, more enzymes can be easily added to replenish the damaged enzyme.

When laser excitation is used to detach the blocking group, the laser 25 can be directed at the molecule and cause the detachment. An example of this is linkage to the nucleoside by a photolabile o-nitrobenzyl ether. Not only can the laser procedure be used to remove the blocking group but it can also be used to activate the reporter and thus measurement of which nucleotide was attached. In one embodiment of the invention the laser excitation method is used because it provides easier automation and decreases the number of reporters required.

Another embodiment of the present invention is the device 52 shown in Figure 3. The device 52 can be used for simultaneously sequencing a plurality of DNA or RNA sequences and comprises a first reservoir 34 for detaching buffer, a second reservoir 37 for a reaction buffer, a third reservoir 31 for a washing buffer, a plurality of columns 12, each column 12 including a solid support 10 for attaching a DNA or RNA template with attached prime There is also included a detector means 22 and a fourth reservoir 40 for waste. In this device the first 34, the second 37 and the third 31 reservoirs are connected to the entrance ends of the columns 12 through a first controllable valve 43 to supply the appropriate buffer to said columns 12. The detector means 22, the fourth reservoir 40 and the second reservoir 37 are connected to the exit end of

the columns 12 through a second controllable valve 46 to direct the flow through the columns 12 through the detector 22 and into the waste reservoir 40 or to recycle the reaction buffer into the reservoir 37. In enhanced embodiments of this device there is also a computer means (not shown) which can be attached to the device. The computer means regulates the sequencing procedure. It can control the first 43 and second 46 controllable valves. Further, it can record the output from the detector means 22, and be used for storage and output. One skilled in the art readily recognizes that a variety of controller boards and personal computers can be used as the computer means.

An alternate device 49 for simultaneously sequencing a plurality of DNA and RNA strands 13 is shown in Figure 5. This device 49 comprises a solid support 10 for attaching the plurality of DNA or RNA templates 13 with annealed primers 16 at discreet locations. An example of a solid support 10 with discreet locations 55 is schematically shown in Figure 6. In Figure 5 a plurality of buffer reservoirs 31, 32, 34 and 37 for holding wash buffers A & B, detaching buffer and reaction buffer, can be seen. Further a detector means 22 for identifying the addition of a nucleotide to the annealed primers is also shown. In the device 49 the solid support 10, buffer reservoirs 31, 32, 34 and 37, and detector means 22 are relationally positioned on a movement means such that there are repeated cycles of the solid support 10 sequentially contacting the (i) reaction buffer to add a modified nucleotide to the primers, (ii) the first wash buffer to remove unreacted nucleotides, (iii) the detector means to identify which nucleotide was added to each primer, (iv) the detaching buffer for removing a blocking group and reporter from the attached modified nucleotide, and (v) the second wash buffer to remove traces of the detaching buffer. One skilled in the art will readily recognize that the first and second wash buffer could be identical and thus only one wash buffer reservoir would be needed. The device 49 schematically shown in

Figure 5 can also further comprise a computer means for automating and regulating the number of repeated cycles and the sequential movement of the support 10, reservoirs 31, 32, 34 and 37, and detector 22. Additionally, the computer means can be attached to the detector 22 in order to record the output of the detector means. This output can then be stored for further use or manipulated and output into more easily readable reports. Additionally the whole device can be incorporated into a cabinet.

In the preferred embodiment of the devices 49 and 52, the solid support is avidin coated glass plates. The biotinylated DNA can then be attached to the avidin surface. The primers can be attached to the DNA prior to attachment to the solid support or can be added after attachment.

In the preferred embodiment of the invention the detector means includes a laser for excitation of the fluorescent reporter groups and a camera and a computer for digitation. One skilled in the art will readily recognize that a variety of detectors are available. Examples include charge coupled device (CCD) camera, video camera with filter, fluorescent microscopes, regular photo multiplier and photographs. The selection of the detector will depend on the reporter used. A fluorescent dye, radioisotope, chemiluminescent compound, antibody, or other reporter/marker can be used. A variety of such devices are available commercially. In the present means the detection means is selected depending on which marker or reporter is used.

The following examples are offered by way of illustration are not intended to limit the invention in any manner.

Example 1

Base Addition Sequencing Scheme (BASS) Analysis of DNA Templates Attached to Solid Supports With a Flow Through Director

The following procedure shows BASS analysis of a single DNA template attached to a solid support. The DNA template is initially bound to a solid support, with a short primer strand concurrently or subsequently annealed to it. This combination is then contacted with the four modified nucleotides and enzyme in the appropriate buffer (25mM Tris, pH 7.8, 50mM NaCl 10mM MgCl, 5-50 units DNA polymerase). Then the primer is enzymatically extended by one base (at 37°C or 50°C for about 2 to 30 mins.). The 3' leaving group (LG) of the modified nucleotide blocks further extension, and Watson - Crick base pairing ensures that only the correct nucleotide is added. Then unreacted nucleotides are washed away. Then the leaving group, which may be a fluorescent moiety with an acid-labile linker, is removed by an acid wash. The 3' end of the nucleotide is now ready for the addition of another modified nucleotide. The leaving group is eluted to a fluorescence detector which can distinguish between the reporter groups, and the reporter is detected.

This scheme makes use of established techniques for the attachment of a single DNA strand (template) to a strepavidin coated solid support via a biotin group at the 3' (or 5') terminus. In figure 2A, the oligonucleotide template is already bound to an oligonucleotide primer. The sequencing begins by mixing the DNA template/primer complex with the four modified nucleotides and the DNA polymerase in an appropriate buffer. One of the four nucleotides will be added to complement the next base in the sequence. In the figure 2B, the modified nucleotide 19 is LG:A (i.e., a modified dATP) which complements the first 'T' in the template sequence. Once the a base is added no further elongation of the primer can occur that cycle due to the presence of the blocking group attached at the 3' terminus.

After the unreacted nucleotides are washed away, in figure 2C the DNA template/primer complex is treated with mild acid to cause the group

blocking the 3' hydroxyl terminus of the primed site to leave, restoring an active 3' hydroxyl site. A fluorescence detector identifies the group as it leaves the column and therefore indicates the previous addition of the modified 'A' nucleotide at the first site in the sequence. The process is repeated with a new base added each time, and the fluorescent detection of a new leaving moiety each cycle. The accumulated record of the different fluorescent groups leaving the column after each cycle represents the DNA sequence of the template strand. It should be noted that a simple way to 'multiplex' the procedure is by having multiple columns with different templates in parallel.

Example 2

BASS Analysis of a DNA Template Bound

To Solid Supports With *In Situ* Detection

BASS analysis of a DNA sequence can also be done with in situ detection of the added bases. The scheme is very similar to that described in Example 1 above. A DNA template is bound to a solid support and a short primer strand annealed to it. This combination is then contacted with the four modified nucleotides and enzyme in the appropriate buffer (see conditions Example 1). Then the primer is enzymatically extended by one base (see conditions Example 1). The 3' leaving group (LG) blocks further extension, and Watson - Crick base pairing ensures that only the correct nucleotide is added. Unreacted nucleotides are washed away. A capping step may be performed now or after the following step. The fluorescent reporter is detected by the detector means while still in place on the template. In this procedure the reporter is excited by an argon laser and the emission spectra is recorded by a fluorescence detector. The detector can distinguish between the reporter groups. Then the leaving group is cleaved with mild acid and eluted off the column, restoring the 3' terminal hydroxyl, and therefore allowing further nucleotide additions.

To facilitate the in situ detection the oligonucleotide (DNA or RNA) can be fixed to a glass plate as a solid support. This greatly facilitates 'multiplex' analysis of DNA templates since an array of different DNAs can be attached to a single glass plate and all the samples treated in parallel. With currently available fluorescent imaging software and hardware very large numbers of DNA templates are attached to single plates. Thus, thousands of sequences are determined in a single BASS run.

Example 3

Application of BASS

BASS is applicable to any situation where other DNA sequence methods can be used. These include:

(a) The analysis of different genes and genomes. The sequencing of the human genome is possible with this method.

(b) The identification and characterization of different organisms.

(c) The diagnosis of genetic diseases and cancers.

BASS also has several advantages over previous sequencing schemes. These include:

(a) Obviating the gel electrophoresis required for other methods, so that size resolution of different fragments does not limit the extent of the sequence that can be obtained in a single analysis,

(b) More signal from each base addition in the scheme is generated than by other methods, since at any one step in the analysis all the DNA templates will be labelled only with a single base. In the Sanger sequencing scheme, for example, all the signal is distributed over all the bases to be analyzed,

(c) BASS is very easily and economically multiplexed so that thousands of DNA templates are simultaneously analyzed with a reduction in cost and effort relative to current methods.

(d) less secondary structural problems.

All publications mentioned in this specification are indicative of the skill level of those skilled in the art to which the invention pertains. All publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The devices, compounds, assays, methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

What is claimed is:

CLAIMS

1. A multiplex assay for determining the sequence of a strand of DNA or RNA, comprising the steps of:

attaching a plurality of DNA or RNA templates with annealed primers to a solid support;

contacting the attached DNA or RNA templates with a reaction buffer, said buffer containing modified nucleotides and an enzyme to attach the modified nucleotides to the 3'-hydroxyl terminus of the primer attached to each DNA or RNA, wherein the modified nucleotides include a blocking group and a reporter, wherein the blocking group blocks the addition of further nucleotides to the primer and the reporter identifies which nucleotide has attached to the primer;

removing unreacted modified nucleotides by washing said solid support with a first wash buffer;

detecting the attached modified nucleotide added to each primer;

removing the blocking group and reporter by contacting the DNA or RNA with a detaching buffer;

contacting the attached DNA or RNA with a second wash buffer to remove traces of the detaching buffer; and

repeating the above steps until the DNA or RNA has been sequenced.

2. The assay of claim 1, wherein the solid support is selected from the group consisting of a Strepavidin/biotin coated surface, a derivatized photoactivatable glass support and a NH_2 binding group attached to glass.

3. The assay of claim 1 wherein the solid support is an avidin coated support;

4. The assay of claim 1, wherein the modified nucleotide includes a reporter selected from the group consisting of metal ions, fluorescent dyes, radioisotopes, antibodies and chemiluminescence compounds.

5. The assay of claim 1, wherein the reporter is a fluorescent dye.

6. The assay of claim 4, wherein each different nucleotide which can be added to the primer has a different reporter.

7. The assay of claim 1, wherein the detecting step includes excitation by laser and recording of the emission spectra.

8. The assay of claim 1, wherein the detection is by laser excitation and CCD camera digitization.

9. The assay of claim 1, wherein the blocking group and reporter are removed prior to the detecting step.

10. The assay of claim 1, wherein the enzyme is selected from the group consisting of T7, Taq, Klenow, T_4, Pol 1, DNA polymerase and reverse transcriptase.

11. The assay of claim 1, wherein the modified nucleotides are 2'—deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine and 2'-deoxythymidine, each with a blocking group and reporter attached to the 3'-OH group of said modified nucleotide.

12. A device for simultaneously sequencing a plurality of DNA or RNA sequences, comprising:

a first reservoir for a detaching buffer;

a second reservoir for a reaction buffer;

a third reservoir for a washing buffer;

a plurality of columns, each column including a solid support for attaching a DNA or RNA template with attached primers;

a detector means; and

a fourth reservoir for waste;

said first, second and third reservoirs being connected to an entrance end of said columns through a first controllable valve to supply an appropriate buffer at appropriate time to said columns;

said detector means, fourth reservoir and second reservoir being connected to an exit end of said columns through a second controllable valve to direct column flow through said detector and into said waste reservoir and to recycle said reaction buffer.

13. The device of claim 12, further comprising a computer means attached to said device for regulating the controllable valves and for recording the output from the detecting means.

14. A device for simultaneously sequencing a plurality of DNA or RNA strands, comprising:

a solid support for attaching the plurality of DNA or RNA templates with annealed primers at discrete locations;

a plurality of buffer reservoirs for holding reaction buffer, wash buffer and detaching buffer;

a detector means for identifying addition of a nucleotide to the annealed primers; and

a movement means, wherein said solid support, buffer reservoirs and detector means are relationally positioned on said movement means such that there are repeated cycles of the solid support with attached DNA or RNA sequentially contacting the reaction buffer to add a modified nucleotide to the primers, a first wash buffer to remove unreacted nucleotides, the detector means to identify which nucleotide was added to each primer, a detaching buffer for removing a blocking group and reporter from the attached modified nucleotide, and a second wash buffer to remove traces of said detaching buffer.

15. The device of claim 14, further comprising a computer means for automating and regulating the number of repeat cycles and the sequential movement, of the support, reservoirs and detector.

16. The device of claim 14, further comprising a computer means for recording the output of the detector means.

17. The device of claim 14, wherein the solid support is an avidin coated glass plate.

18. The device of claim 14, wherein the detector means includes a laser for excitation and CCD camera for digitization.

19. The device of claim 14, wherein the first and second wash buffer reservoirs are combined to form a single reservoir.

20. The device of claim 14, further including a cabinet to hold the device.

21. A device for the automated sequencing of a plurality of DNA or RNA strands, comprising:

a solid support for attaching the plurality of DNA or RNA templates with annealed primers at discreet locations;

a plurality of buffer reservoirs for holding reaction buffer, wash buffer and detaching buffer;

a detector means for identifying addition of a nucleotide to the annealed primers;

a movement means, wherein said solid support, buffer reservoirs and detector means are relationally positioned on said movement means such that there are repeated cycles of the solid support with attached DNA or RNA sequentially contacting the reaction buffer to add a modified nucleotide to the primers, a first wash buffer to remove unreacted nucleotides, the detector means to identify which nucleotide was added to each primer, a detaching buffer for removing a blocking group and reporter from the

attached modified nucleotide and a second wash buffer to remove traces of the detaching buffer; and

a computer means, wherein the computer means is connected to said movement means to regulate the number of repeated cycles and the sequential movement, and the computer means is attached to the detector means to record the output.

22. The device of claim 21, further including a cabinet for holding all the elements together.

23. The device of claim 21, wherein the first and second wash buffer reservoirs are combined to form a single reservoir.

24. A method for determining the sequence of an oligonucleotide template comprising the steps of:

attaching a primed template to a solid support;

contacting said primed template with a reaction buffer containing a modified nucleotides, and an enzyme to add one of said modified nucleotide to a 3'-hydroxyl terminus of said primer of said primed template, wherein said modified nucleotides include a blocking group and a reporter;

adding one of said modified nucleotides to said 3'-hydroxyl terminus;

washing said primed template with a first wash buffer;

removing said blocking group and reporter from said added modified nucleotide by contacting said primed template with a detaching buffer;

detecting said reporter in said detaching buffer after removing said reporter from said added modified nucleotide;

washing said primed template with a second wash buffer; and

repeating above said steps until said oligonucleotide template is sequenced.

25. The method of claim 24, wherein an oligonucleotide template is attached to said solid support in said attaching step, and a primer is annealed to said template to form a primed template;

26. The method of claim 24, further comprising a decontaminating step practiced concurrent with or immediately after said contacting step.

27. The method of claim 24, further comprising a capping step practiced after said adding step and before said washing step.

28. The method of claim 24, further comprising a capping step practiced after said adding step and before said washing step, wherein a capping buffer is used which contains a capping agent having a non-labile, in situ reporter.

26. A procedure for automating the base addition sequencing scheme comprising the steps of:

attaching an oligonucleotide template to a solid support;

annealing a primer to said template to form a primed template;

contacting said primed template with a reaction buffer containing a modified nucleotides, and an enzyme to add one of said modified nucleotide to a 3'-hydroxyl terminus of said primer of said primed template, wherein said modified nucleotides include a blocking group and a reporter;

adding one of said modified nucleotides to said 3'-hydroxyl terminus;

capping any unreacted primed templates with a capping agent having an in situ detectable marker;

washing said primed template with a first wash buffer;

removing said blocking group and reporter from said added

modified nucleotide by contacting said primed template with a detaching buffer;

detecting said reporter in said detaching buffer after removing said reporter from said added modified nucleotide;

washing said primed template with a second wash buffer; and

repeating above said steps until said oligonucleotide template is sequenced.

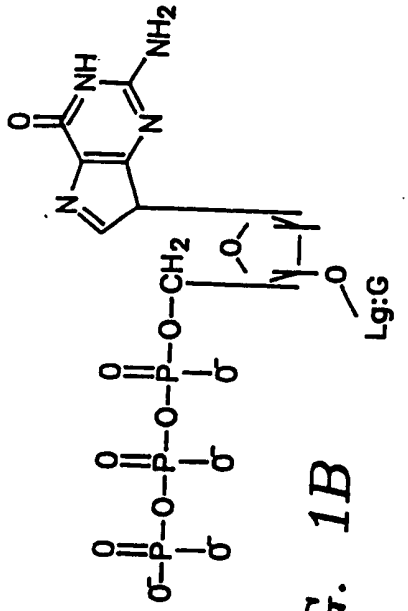

FIG. 1B

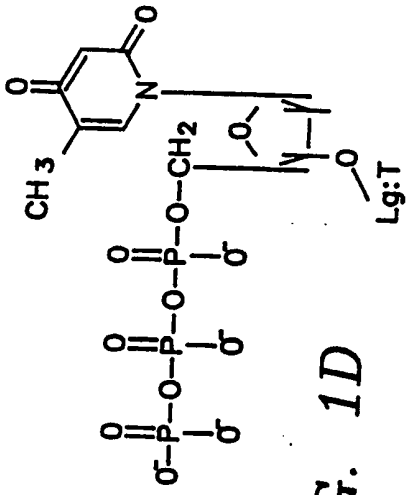

FIG. 1D

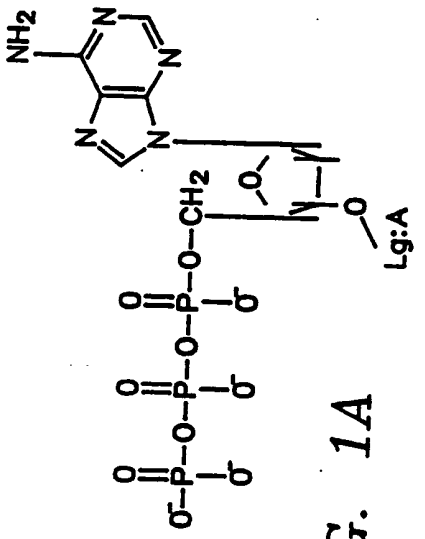

FIG. 1A

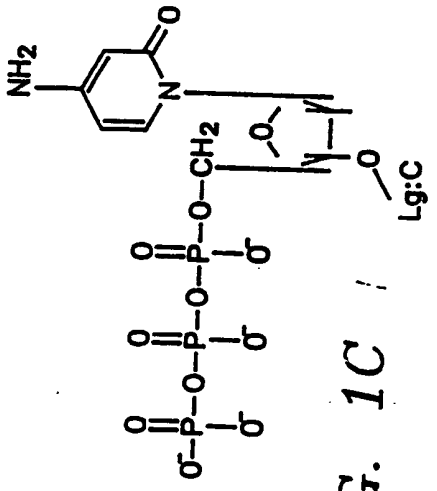

FIG. 1C

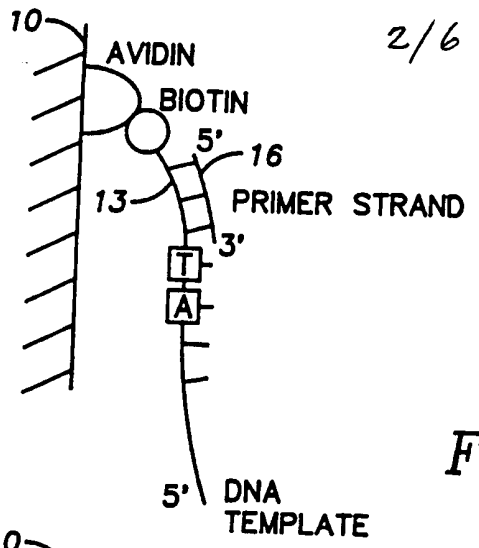

FIG. 2A

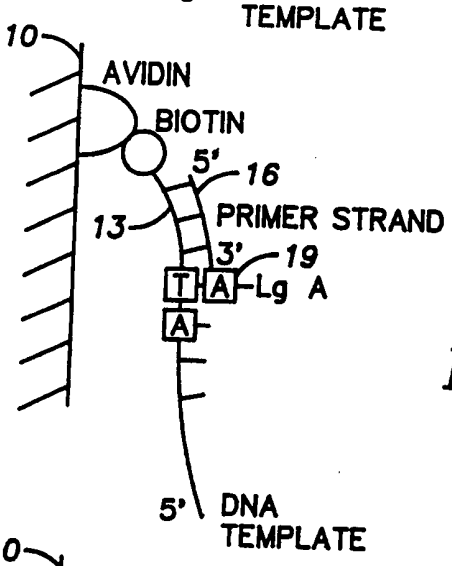

FIG. 2B

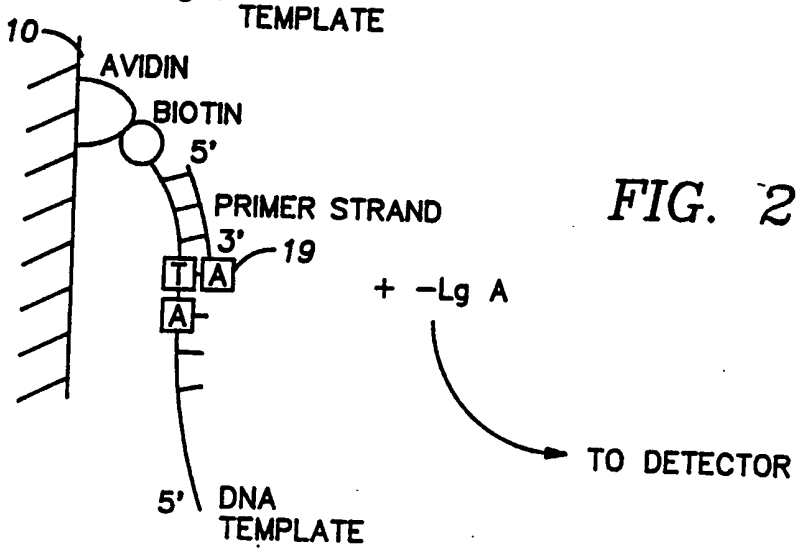

FIG. 2C

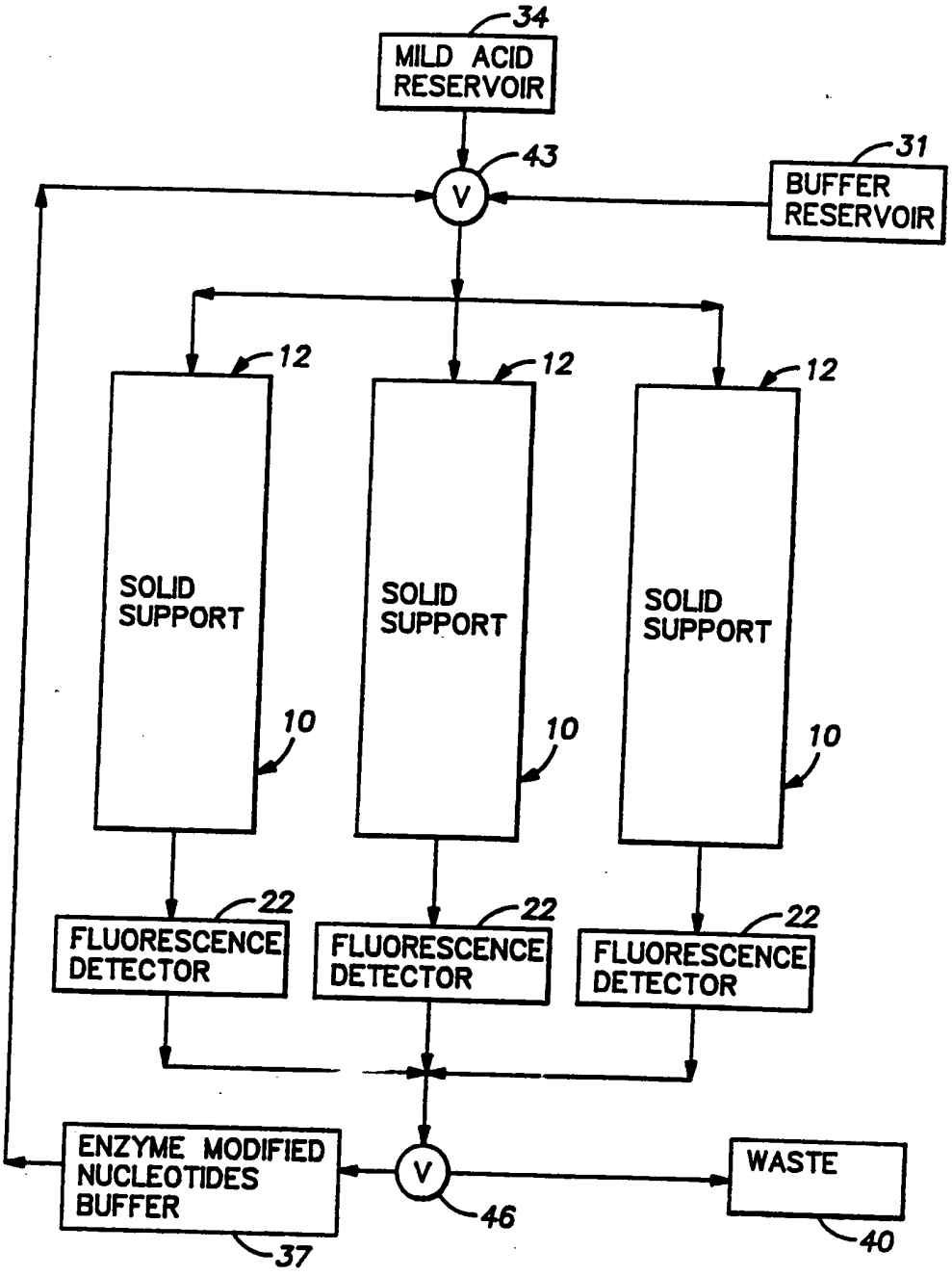
34
MILD ACID RESERVOIR
43
V
31
BUFFER RESERVOIR
12
12
12
SOLID SUPPORT
SOLID SUPPORT
SOLID SUPPORT
10
10
10
22
22
22
FLUORESCENCE DETECTOR
FLUORESCENCE DETECTOR
FLUORESCENCE DETECTOR
ENZYME MODIFIED NUCLEOTIDES BUFFER
37
V
46
WASTE
40

FIG. 3

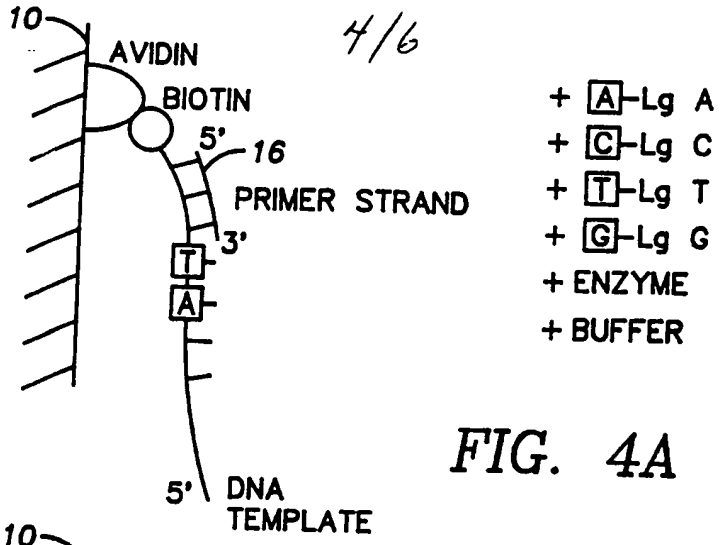

FIG. 4A

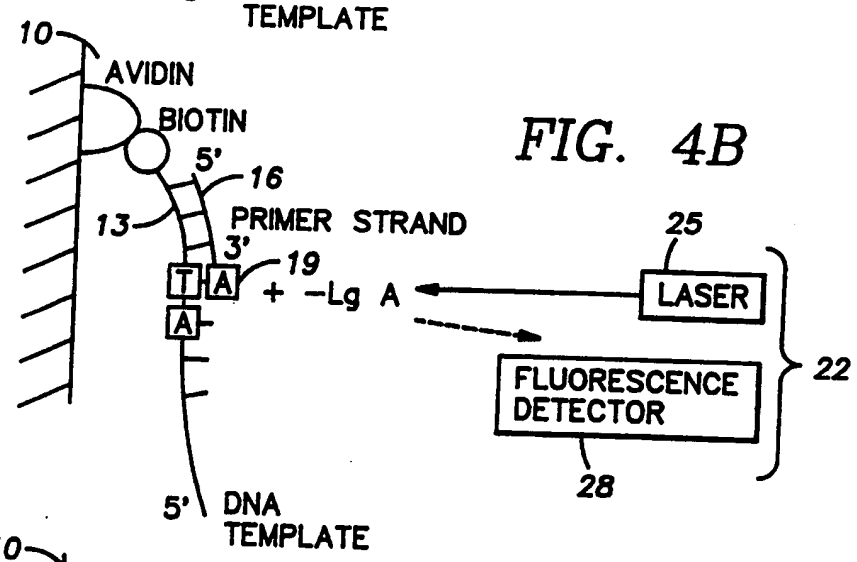

FIG. 4B

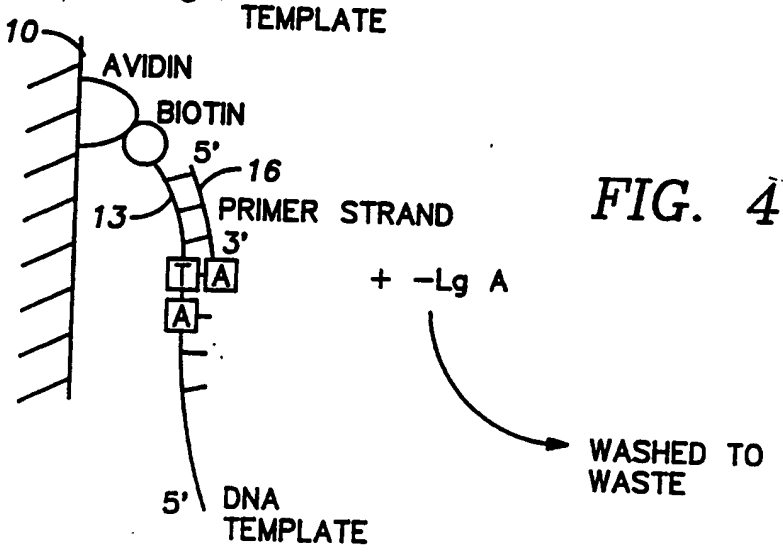

FIG. 4C

5/6

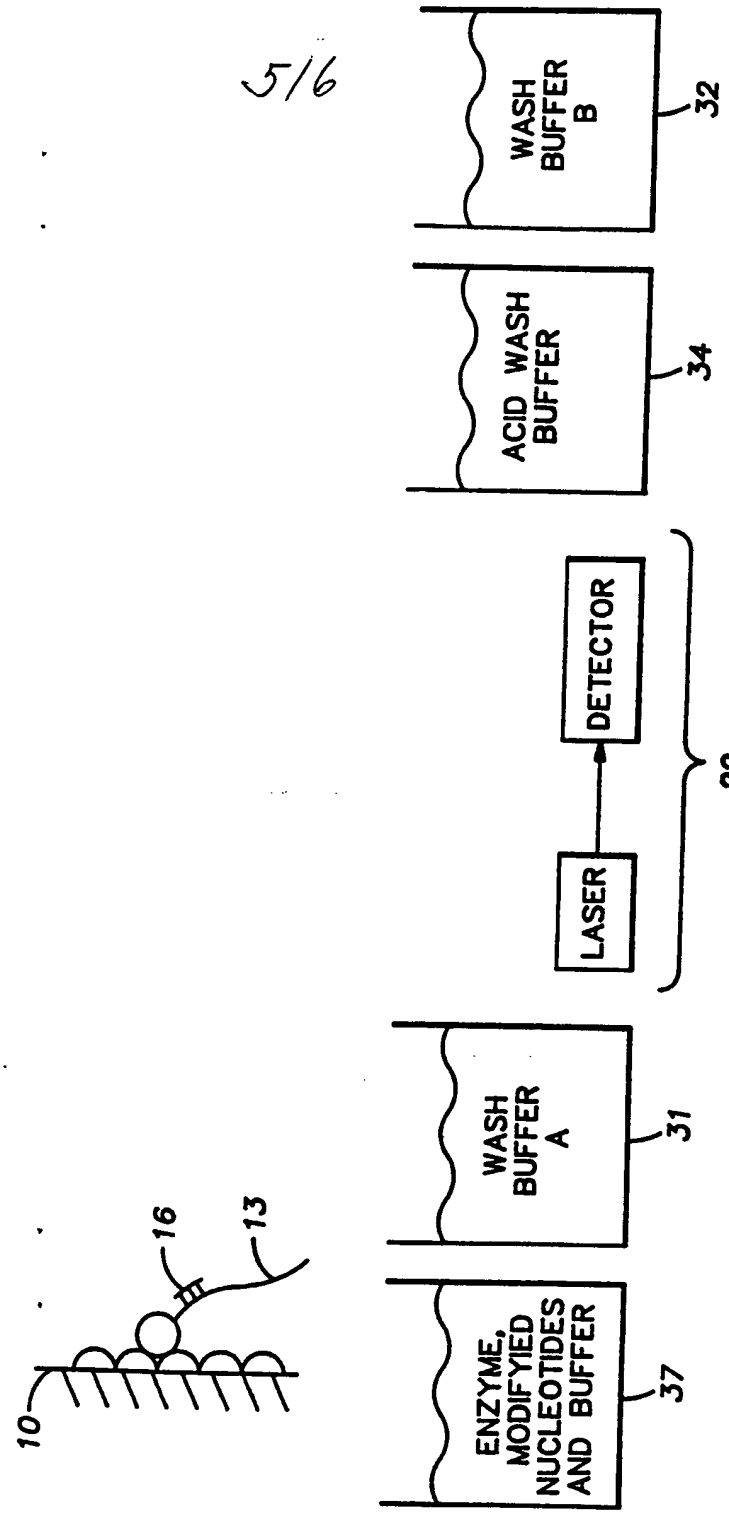
WASH BUFFER B
32
ACID WASH BUFFER
34
DETECTOR
LASER
22
WASH BUFFER A
31
ENZYME, MODIFYIED NUCLEOTIDES AND BUFFER
37
16
13
10

FIG. 5

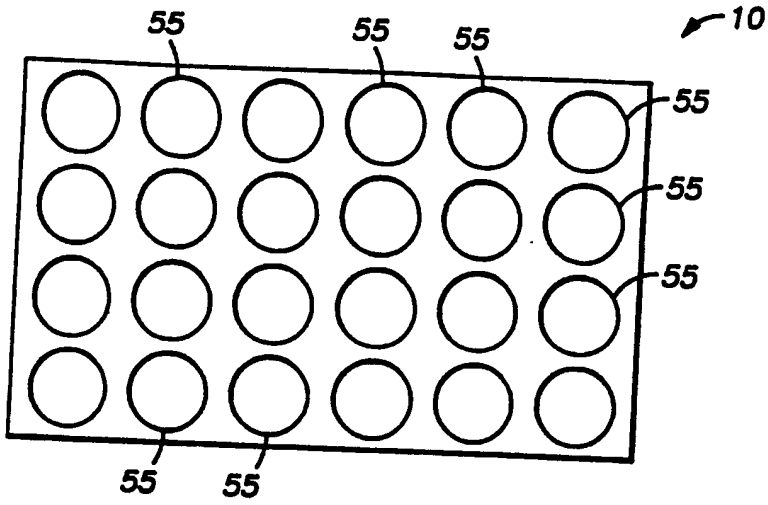

FIG. 6

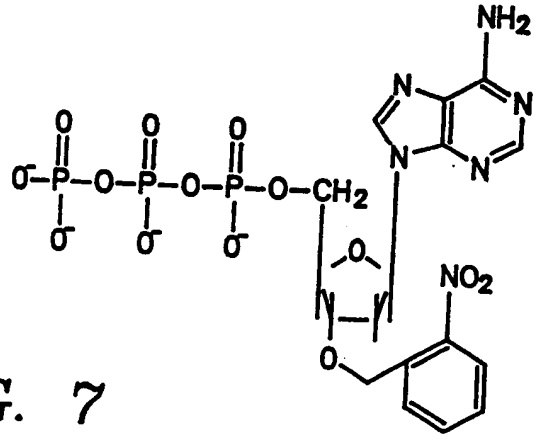

FIG. 7

INTERNATIONAL SEARCH REPORT

Inte.national application No.
PCT/US92/07678

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,673,657 (CHRISTIAN) 16 JUNE 1987, see entire document.	12-23
A	US, A, 4,942,124 (CHURCH) 17 JUNE 1990, see entire document.	1-11, 24-29

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